

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
3 May 2001 (03.05.2001)

PCT

(10) International Publication Number
WO 01/30320 A1

- (51) International Patent Classification⁷: A61K 9/16. (74) Agents: ODRE, Steven, M. et al.; Amgen Inc., One Amgen Center Drive, M/S 27-4-A, Thousand Oaks, CA 91320-1799 (US).
- (21) International Application Number: PCT/US00/29257 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 23 October 2000 (23.10.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
09/426,566 22 October 1999 (22.10.1999) US
Not furnished 13 October 2000 (13.10.2000) US
- (71) Applicant: AMGEN INC. [US/US]; One Amgen Center Drive, Thousand Oaks, CA 91320-1799 (US).
- (72) Inventors: BURKE, Paul; 261 Melrose Drive, Oxnard, CA 93035 (US). KLUMB, Lisa; 124 Eagle Rock Avenue, Oxnard, CA 93035 (US). MURPHY, Keith; 922 14th Street #210, Santa Monica, CA 90403 (US). HERBERGER, John; 4009 Willow Creek Lane, Moorpark, CA 93021 (US). FRENCH, Donna, L.; 11867 Tuscan Court, Moorpark, CA 93021 (US).
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— With international search report.
— Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



BEST AVAILABLE COPY

WO 01/30320 A1

(54) Title: BIODEGRADABLE MICROPARTICLES WITH NOVEL ERYTHROPOIETIN STIMULATING PROTEIN

(57) Abstract: The present invention relates to a composition for the sustained release of biologically active, novel erythropoietin stimulating protein (NESP), and improved methods of forming said composition. The composition comprises polymeric microparticles within which particles of NESP have been dispersed. The improved method utilizes a cosolvent mixture to effect more efficient and rapid removal of residual polymer solvents during any drying process.

BIODEGRADABLE MICROPARTICLES WITH NOVEL ERYTHROPOIETIN STIMULATING PROTEIN

FIELD OF THE INVENTION

5

The present invention relates to a composition for the sustained release of biologically active, novel erythropoietin stimulating protein (NESP), and improved methods of forming said composition. The composition comprises polymeric microparticles within which particles of NESP have been dispersed. The improved method utilizes a cosolvent mixture to effect more efficient and rapid removal of residual polymer solvents during any drying process.

15

BACKGROUND OF THE INVENTION

For the past several years, there have been extensive efforts directed to the development of effective sustained-release formulations which could provide a means of controlling blood levels of the active ingredient, and also provide greater efficacy, safety, patient convenience and patient compliance. Unfortunately, the instability of most proteins (e.g. denaturation and loss of bioactivity upon exposure to heat, organic solvents, etc.) has greatly limited the development and evaluation of sustained-release formulations.

Methods of preparing microparticles in the prior art have been described in both the patent and scientific literature. In particular, various methods are described for preparing biodegradable microparticles of poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) for controlled release of water-soluble drugs; see e.g., Wise et al., *Contraception*, 8:227-234

-2-

(1973); Hutchinson et al., *Biochem. Soc. Trans.*, 13:520-523 (1985); and Jalil et al., *J. Microencapsul.*, 7:297-325 (1990); Putney et al., *Nature Biotech.*, 16:153-157 (1998); Burke, P., *Handbook of Pharmaceutical Controlled Release Technology*, Klibanov, et al. (editors) (in press). These methods include those involving emulsions (phase separation, solvent extraction and solvent evaporation) and those involving atomization (spray drying, spray freezing).

The major disadvantages associated with the above-referenced methods may include, under certain circumstances: 1) high temperatures causing protein inactivation; 2) exposure to organic solvents causing protein inactivation; 3) inability to encapsulate hydrophilic drugs due to loss of drug to the aqueous phase which is used to extract the organic solvent; 4) the large amount of organic solvents normally needed during the processes, and which cannot be adequately removed from the final product, i.e., high residual solvent levels in final product; 5) poor protein loading efficiencies; 6) poor overall yields; 7) problems with drug leakage and/or high initial drug release upon administration; and 8) expensive and complex to scale-up. As specifically relates to item 4), the problems associated with high levels of solvents in spray drying processes have been described; see e.g., Clarke et al., *Drug Devel. and Industrial Pharmacy*, 24:169-175 (1998); Bitz and Doelker, *Inter. Journal of Pharm.*, 131:171-181 (1996); and Takada et al., *Journ. of Controlled Release*, 32:79-85 (1994).

There have been numerous reports in both the patent and scientific literature regarding improved processes for microparticle preparation. For example, in Gombotz et al., U.S. Patent No. 5,019,400, a process is disclosed for preparing microparticles wherein very

-3-

cold temperatures are used to freeze polymer-biologically active agent mixtures into polymeric microspheres with high retention of biological activity and material. In Ramstack et al., U.S. Patent No. 5,650,173, a process is disclosed for preparing microparticles wherein a blend of at least two non-toxic solvents, free of halogenated hydrocarbons, was used to dissolve the polymer and the active agent. The resulting microparticles, though free of residual toxic solvents, still had residual amounts of benzyl alcohol and ethyl acetate, which had a negative effect on product integrity. In Rickey et al., U.S. Patent No. 5,792,477, a process was described which alleviated the residual solvent problem reported in Ramstack et al., by including additional washing steps in the process to effect adequate removal of solvents.

While these and other research efforts have clearly furthered the technology, there still exists a need for a more efficient, economical, broadly applicable microparticle preparation process for use with proteins, peptides, and small molecules, that is amenable to aseptic processing and is scaleable.

Among the various proteins for which an effective sustained release composition has been reported is erythropoietin. Erythropoietin (EPO) is a glycoprotein hormone involved in the maturation of erythroid progenitor cells into erythrocytes. It is produced in the kidney and is essential in regulating levels of red blood cells in the circulation. Conditions marked by low levels of tissue oxygen signal increased production of erythropoietin, which in turn stimulates erythropoiesis. A loss of kidney function as is seen in chronic renal failure (CRF), for example, typically results in decreased production of

-4-

erythropoietin and a concomitant reduction in red blood cells.

Administration of recombinant human erythropoietin (rHuEPO) is effective in raising red blood cell levels in anemic patients with end stage renal disease; Eschbach et al., *New Eng. J. Med.*, 316:73-38 (1987). Subsequent studies have shown that treatment with rHuEPO can correct anemia associated with a variety of other conditions; Fischl et al., *New Eng. J. Med.*, 322:1488-1493 (1990); Laupacis, *Lancet*, 341:1228-1232 (1993). Regulatory approvals have been given for the use of recombinant human erythropoietin in the treatment of anemia associated with CRF, anemia related to therapy with AZT (zidovudine) in HIV-infected patients, anemia in patients with non-myeloid malignancies receiving chemotherapy, and anemia in patients undergoing surgery to reduce the need of allogenic blood transfusions. Current therapy for all approved indications (except the surgery indication) involves a starting dose of between 50-150 Units/kg three times per week (TIW) administered either by an intravenous (IV) or subcutaneous (SC) injection to reach a suggested target hematocrit range of 30% to 36%. For the surgery indication, rHuEPO is administered every day 10 days prior to surgery, on the day of surgery, and four days thereafter (EPOGEN® Package Insert, 12/23/96). In general, the current recommended starting doses for rHuEPO raise hematocrit into the target range in about six to eight weeks. Once the target hematocrit range has been achieved, a maintenance dosing schedule is established which will vary depending upon the patient, but is typically three times per week for anemic patients with CRF. The administration of rHuEPO described above is an effective and well-tolerated regimen for the treatment of anemia.

-5-

In Zale et al., U.S. Patent No. 5,674,534, sustained-release compositions of non-aggregated, biologically active EPO are described. The compositions comprise a polymeric matrix of a biocompatible polymer and particles of biologically active, aggregation-stabilized EPO, wherein said particles are dispersed within the biocompatible polymer, and wherein said microparticles are prepared using the process described in U.S. Patent No. 5,019,400. The method described and claimed utilizes a salting-out excipient to stabilize the EPO. The formulation is said to have advantages including longer, more consistent *in vivo* blood levels of EPO, lower initial bursts of EPO, and increased therapeutic benefits by eliminating fluctuations in serum EPO levels.

Recombinant human erythropoietin expressed in mammalian cells contains three N-linked and one O-linked oligosaccharide chains which together comprise about 40% of the total molecular weight of the glycoprotein. N-linked glycosylation occurs at asparagine residues located at positions 24, 38 and 83 while O-linked glycosylation occurs at a serine residue located at position 126; Lai et al., *J. Biol. Chem.*, 261:3116 (1986); Broudy et al., *Arch. Biochem. Biophys.*, 265:329 (1988). The oligosaccharide chains have been shown to be modified with terminal sialic acid residues. Enzymatic treatment of glycosylated erythropoietin to remove sialic acid residues results in a loss of *in vivo* activity but does not affect *in vitro* activity; Lowy et al., *Nature*, 185:102 (1960); Goldwasser et al., *J. Biol. Chem.*, 249:4202 (1974). This behavior has been explained by rapid clearance of asialo-erythropoietin from the circulation upon interaction with the hepatic asialoglycoprotein binding protein; Morrell et al., *J. Biol. Chem.*, 243:155 (1968); Briggs, et al.,

-6-

Am. J. Physiol., 227:1385 (1974); Ashwell et al.,
Methods Enzymol., 50:287 (1978).

Novel erythropoietin stimulating protein
(NESP) is a hyperglycosylated erythropoietin analog
5 having five changes in the amino acid sequence of rHuEPO
which provide for two additional carbohydrate chains.
More specifically, NESP contains two additional N-linked
carbohydrate chains at amino acid residues 30 and 88
(numbering corresponding to the sequence of human
10 EPO) (see PCT Application No. US94/02957, herein
incorporated by reference in its entirety). NESP is
biochemically distinct from EPO, having a longer serum
half-life and higher in vivo biological activity; Egrie
et al., ASH 97, Blood, 90:56a (1997). NESP has been
15 shown to have ~3 fold increase in serum half-life in
mice, rats, dogs and man; Id. In mice, the longer serum
half-life and higher in vivo activity allow for less
frequent dosing (once weekly or once every other week)
compared to rHuEPO to obtain the same biological
20 response; Id.

A pharmacokinetic study demonstrated that,
consistent with the animal studies, NESP has a
significantly longer serum half-life than rHuEPO in
chronic renal failure patients, suggesting that a less
25 frequent dosing schedule may also be employed in humans;
MacDougall, et al., J American Society of Nephrology,
8:268A (1997). A less frequent dosing schedule would be
more convenient to both physicians and patients, and
would be particularly helpful to those patients involved
30 in self-administration. Other advantages to less
frequent dosing may include less drug being introduced
into patients, a reduction in the nature or severity of
the few side-effects seen with rHuEPO administration,
and increased compliance.

-7-

The present invention is based upon the discovery that NESP can be encapsulated in microparticles and provide a pharmaceutical composition with an even more dramatic sustained release profile, allowing for a once every 4-6 week dosing for raising hematocrit and treating anemia, and thus providing tremendous therapeutic advantage. Additionally, the NESP/PLGA system is such that the PLGA component is cleared (biodegraded) up to at least one week prior to the cessation of therapeutic effect, and thus allow for repeated dosing with less concern of polymer and drug build-up from dose to dose. Such a system may not be possible with, for example, EPO/PLGA microparticles.

Additionally, the present invention provides an improved, economical, scalable method for microparticle preparation which is broadly applicable to biologically active agents other than NESP, including peptides and small molecules.

SUMMARY OF THE INVENTION

Accordingly, one aspect of the present invention is a pharmaceutical composition for the sustained-release of an active ingredient comprising a biologically active ingredient contained within polymeric microparticles. Importantly, the sustained-release compositions of the present invention maintain the activity, integrity and safety of the active ingredient during encapsulation and release, which helps to provide for longer periods of consistent release.

A second aspect of the present invention relates to a new and improved process for preparing a sustained-release composition comprising an active ingredient contained within polymeric microparticles. The process is economical, amenable to aseptic

-8-

processing, scalable and broadly applicable to proteins, peptides, and small molecules. The improved process can be generally described as comprising the steps of: (a) obtaining a dried powder of a specific active ingredient
5 or formulated active ingredient; (b) preparing a polymer solution comprising a polymer dissolved in a cosolvent mixture; (c) dispersing said dried powder in said polymer solution to produce an active ingredient/polymer mixture; (d) preparing active
10 ingredient-containing microparticles from said mixture; (e) collecting said microparticles; and (f) finishing said microparticles by secondary drying. Importantly, the drying times associated with this process are significantly reduced as compared to prior art methods.

15

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic of the process of the present invention for making the active ingredient
20 containing microparticles.

Figure 2 shows chromatograms depicting the size exclusion chromatography results for a NESP preparation prior to spray drying (___ . . .), a spray
25 dried NESP protein powder (___), and a typical result for NESP after microparticle encapsulation (___).

Figure 3 is a graph depicting NESP serum levels for various microparticle preparations which had
30 been injected subcutaneously (360 µg/kg NESP peptide dose) into rats. Serum concentration is plotted vs. time (days).

-9-

Figure 4 is a graph depicting hematocrit for rats injected subcutaneously (360 $\mu\text{g/kg}$ NESP peptide dose) with various microparticle preparations. Hematocrit (%) is plotted vs. time (days).

5

Figure 5 is a graph depicting NESP serum levels for various microparticle preparations which had been injected subcutaneously (360 $\mu\text{g/kg}$ NESP peptide dose) into rats. Serum concentration is plotted vs. time (days).

10

Figure 6 is a graph depicting NESP serum levels for various microparticle preparations which had been injected subcutaneously (360 $\mu\text{g/kg}$ NESP peptide dose) into rats. Serum concentration is plotted vs. time (days).

15

Figure 7 is a graph depicting hemoglobin levels for rats injected subcutaneously with a 10,000 $\mu\text{g/kg}$ bolus injection of NESP vs. a 100 $\mu\text{g/kg}$ (20 mg microparticle) injection of a NESP microparticle preparation (50:50, inherent viscosity 0.4 dL/g). Hemoglobin levels (g/dL) is plotted vs. time (days).

20

Figure 8 is a graph depicting NESP serum levels for various microparticle preparations which had been injected subcutaneously (360 $\mu\text{g/kg}$ NESP peptide dose) into rats. Serum concentration is plotted vs. time (days).

25

30

Figure 9 is a graph depicting leptin serum levels for rats injected with various leptin preparations. Serum concentration is plotted vs. time (hours).

-10-

Figure 10 is a graph depicting % weight loss for rats injected with various leptin preparations. Body weight (mg) is plotted vs. time (days).

5

Figure 11 is a graph depicting NESP serum levels for microparticle preparations which had been injected subcutaneously (360 µg/kg NESP peptide dose) into rats. Serum concentration is plotted vs. time (days).

10

Figure 12 is a graph depicting hematocrit for rats injected subcutaneously (360 µg/kg NESP peptide dose) with microparticle preparations. Hematocrit (%) is plotted vs. time (days).

15

Figure 13 is a graph depicting NESP serum levels for microparticle preparations which had been injected subcutaneously (360 µg/kg NESP peptide dose) into rats. Serum concentration is plotted vs. time (days).

20

Figure 14 is a graph depicting hematocrit for rats injected subcutaneously (360 µg/kg NESP peptide dose) with microparticle preparations. Hematocrit (%) is plotted vs. time (days).

25

DETAILED DESCRIPTION

30

Unless otherwise noted, the term microparticles can be used to encompass microparticles, microspheres, and microcapsules.

-11-

As fully described below, the present invention provides a pharmaceutical composition for the sustained-release of an active ingredient comprising a biologically active ingredient contained within
5 polymeric microparticles. A sustained-release composition is defined as a release of biologically active agent which results in measurable serum levels of said agent for a period of time longer than that obtained following direct administration of aqueous
10 biologically active agent. The sustained release can be continuous or discontinuous, linear or non-linear, and this can be accomplished using one or more polymer compositions, drug loadings, selection of excipients, or other modifications. In one embodiment of the present
15 invention, the sustained-release composition will comprise the biologically active ingredient, NESP.

NESP of the present invention is a hyperglycosylated EPO analog comprising two additional glycosylation sites with an additional carbohydrate
20 chain attached to each site. NESP was constructed using site-directed mutagenesis and expressed in mammalian host cells. Details of the production of NESP are provided in co-owned PCT Application No. US94/02957. New N-linked glycosylation sites for rHuEPO were
25 introduced by alterations in the DNA sequence to encode the amino acids Asn-X-Ser/Thr in the polypeptide chain. DNA encoding NESP was transfected into Chinese Hamster Ovary (CHO) host cells and the expressed polypeptide was analyzed for the presence of additional carbohydrate
30 chains. In a preferred embodiment, NESP will have two additional N-linked carbohydrate chains at residues 30 and 88. The numbering of the amino acid sequence is that of human erythropoietin (EPO). The amino acid sequence of EPO is that depicted in SEQ ID NO: 1. The
35 amino acid sequence of NESP is that depicted in SEQ ID

-12-

NO: 2. It is understood that NESP will have the normal complement of N-linked and O-linked glycosylation sites in addition to the new sites.

The NESP of the present invention may also
5 include conservative amino acid changes at one or more residues in SEQ ID NO: 2. These changes do not result in addition of a carbohydrate chain and will have little effect on the biological activity of the analog.

Other active ingredients to be incorporated
10 into the microparticles of the present invention are synthetic or natural compounds which demonstrate a biological effect when introduced into a living creature. Contemplated active agents include peptides, small molecules, carbohydrates, nucleic acids, lipids,
15 proteins, and analogs thereof. Proteins contemplated for use include potent cytokines, including various hematopoietic factors such as G-CSF, GM-CSF, M-CSF, MGDF, the interferons (alpha, beta, and gamma), interferon consensus, the interleukins (1-12),
20 erythropoietin (EPO), fibroblast growth factor, KGF, TNF, TNFbp, IL-1ra, stem cell factor, nerve growth factor, GDNF, BDNF, NT3, platelet-derived growth factor, and tumor growth factor (alpha, beta), osteoprotegerin (OPG), NESP, and OB protein. OB protein will also be
25 referred to as leptin.

Also contemplated for incorporation into the compositions of the present invention are derivatives, fusion proteins, conjugates, analogs or modified forms of the natural active ingredients. Chemical
30 modification of biologically active proteins has been found to provide additional advantages under certain circumstances, such as increasing the stability and circulation time of the therapeutic protein and decreasing immunogenicity. For example, Davis et al.,
35 U.S. Patent No. 4,179,337, discloses conjugation of

-13-

water-soluble polypeptides such as enzymes and insulin to polyethylene glycol (PEG); see also Kinstler et al., U.S. Patent No. 5,824,784.

In general, comprehended by the present invention are pharmaceutical compositions comprising effective amounts of protein or derivative products of the invention together with pharmaceutically acceptable diluents, stabilizers, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Polysorbate 20, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); see, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. An effective amount of active ingredient is a therapeutically, prophylactically, or diagnostically effective amount, which can be readily determined by a person skilled in the art by taking into consideration such factors as body weight, age, therapeutic or prophylactic or diagnostic goal, and release rate desired.

As used herein, and when contemplating NESP-containing microparticles, the term "therapeutically effective amount" refers to an amount which gives an increase in hematocrit that provides benefit to a patient. The amount will vary from one individual to another and will depend upon a number of factors, including the overall physical condition of the patient and the underlying cause of anemia. For example, a therapeutically effective amount of rHuEPO for a patient

-14-

suffering from chronic renal failure is 50 to 150 units/kg three times per week. The amount of rHuEPO used for therapy gives an acceptable rate of hematocrit increase and maintains the hematocrit at a beneficial level (usually at least about 30% and typically in a range of 30% to 36%). A therapeutically effective amount of the present compositions may be readily ascertained by one skilled in the art using publicly available materials and procedures.

10 The invention provides for administering NESP-containing microparticles less frequently than NESP and/or EPO. The dosing frequency will vary depending upon the condition being treated, but in general will be about one time per 4-6 weeks. It is understood that the dosing frequencies actually used may vary somewhat from the frequencies disclosed herein due to variations in responses by different individuals to the NESP-containing microparticles; the term "about" is intended to reflect such variations.

20 The present invention may thus be used to stimulate red blood cell production and correct depressed red cell levels. Most commonly, red cell levels are decreased due to anemia. Among the conditions treatable by the present invention include anemia associated with a decline or loss of kidney function (chronic renal failure), anemia associated with myelosuppressive therapy, such as chemotherapeutic or anti-viral drugs (such as AZT), anemia associated with the progression of non-myeloid cancers, and anemia associated with viral infections (such as HIV). Also treatable are conditions which may lead to anemia in an otherwise healthy individual, such as an anticipated loss of blood during surgery. In general, any condition treatable with rHuEPO may also be treated with the NESP-containing microparticles of the invention.

-15-

The invention also provides for administration of a therapeutically effective amount of iron in order to maintain increased erythropoiesis during therapy. The amount to be given may be readily determined by one skilled in the art based upon therapy with rHuEPO.

The present invention also provides an improved method for preparing polymeric microparticles containing an active ingredient, comprising utilization of a cosolvent mixture to effect more rapid removal of organic solvents during drying. The residual presence of secondary organic solvent components in the mixture during drying results in acceleration of the removal of residual polymer solvent during any drying process. This improved method provides several significant advantages over the processes described in the art, including, for example, 1) reduction of levels of residual solvents in the polymer system after the initial microparticle formation step; 2) reduction in drying cycle times for polymer systems; and 3) enabling the removal of toxic solvents to acceptable levels for use in human pharmaceuticals. Importantly, these advantages help make such processes commercially practical.

The principal embodiment of the improved method for making the protein loaded microparticles comprises: (a) obtaining a dried powder of an active ingredient or formulated active ingredient, (b) dissolving a polymer in a cosolvent mixture to produce a polymeric solution; (c) adding said dried powder to said polymeric solution to produce a active ingredient/polymer mixture; (d) spray drying said mixture to produce the desired active ingredient loaded microparticles; (e) collecting said microparticles; and (f) finishing said microparticles by secondary drying. The process is shown schematically in Figure 1.

-16-

In one embodiment of the present invention, the active ingredient or formulated active ingredient will be in the form of a spray dried powder. Spray drying is a process wherein a solution is atomized to form a fine mist and dried by direct contact with hot carrier gases. For a detailed review of spray drying, see e.g., Masters, K., "Spray Drying Handbook" (John Wiley & Sons, eds., New York 1984). Provided herein is an improved commercial scale spray dried protein powder preparation method which results in significantly higher yields and improved collection of the microparticles.

Polymer solvents contemplated for use in step b) of the present processes include, for example, chloroform, ethyl acetate, acetone, methylene chloride, and dimethylsulfoxide. In one embodiment of the present invention, the polymer solvent to be used is methylene chloride. Non-solvents contemplated for use include ethanol, ethyl formate, and heptane.

The microparticle preparation step (d) may alternatively involve an emulsion based preparation, or spray freezing. For the emulsions produced in the processes of the present invention, the organic:aqueous ratios contemplated for use are 1:1 to 12:1. In general, the microparticles prepared by the methods of the present invention will generally comprise 0.001-60% by weight of protein.

Secondary drying processes contemplated for use in step (f) include gas bleed drying, fluidized bed drying, lyophilization, vacuum drying, and tray drying. In one embodiment of the present invention, gas bleed drying is utilized in step (f).

Polymers contemplated for use may be selected from the group consisting of biocompatible and/or biodegradable polymers. As defined herein, biodegradable means that the composition will erode or

-17-

degrade in vivo to form smaller biocompatible chemical species. Degradation may occur, for example, by enzymatic, chemical or physical processes. Suitable biodegradable polymers contemplated for use in the present invention include poly(lactide)s, poly(glycolide)s, poly(lactic acid)s, poly(glycolic acid)s, polyanhydrides, polyorthoesters, polyetheresters, polycaprolactone, polyesteramides, polyphosphazenes, polyphosphoesters, pseudo-polyamino acids; Langer, Nature, 392:5-10 (1998), blends and copolymers thereof.

The range of molecular weights contemplated for the polymers to be used in the present processes can be readily determined by a person skilled in the art based upon such factors the desired polymer degradation rate. Typically, the range of molecular weight will be 2000 to 2,000,000 Daltons. Almost any type of polymer can be used provided the appropriate solvent or cosolvent system are found.

The term "PLGA" as used herein is intended to refer to a polymer of lactic acid alone, a polymer of glycolic acid alone, a mixture of such polymers, a copolymer of glycolic acid and lactic acid, a mixture of such copolymers, or a mixture of such polymers and copolymers. PLGA's used may be in the free acid ("uncapped") form or in the terminal ester ("capped") form. Preferably, the biodegradable polymer will be poly lactide-co-glycolide (PLGA). The polymer concentrations contemplated for use in the processes of the present invention are in the range of 5-70 g/100mL (g%).

In general, an aqueous solution, a suspension, or a solid form of the active agent can be admixed with the organic solvent containing the polymer. When an aqueous solution of active ingredient is used,

-18-

an aqueous solution of active ingredient-in-polymer solution emulsion, or a water-in-oil emulsion is formed (containing the active ingredient in the aqueous phase and the polymer in the organic phase) and used to
5 prepare microparticles. When a suspension or solid form of active ingredient is used, suspensions of the solid active ingredient in the polymer solution are formed and used to prepare the microparticles. Alternatively, a monophasic solution of active ingredient and polymer may
10 be used. In one embodiment of the present invention, the active ingredient will be in the form of a spray dried powder, the particle size of the protein powder will be in the range of $<10\ \mu\text{m}$. The protein concentrations contemplated for use in the processes of
15 the present invention are in the range of 0.001-500 mg/mL when in the emulsion or suspension.

The active ingredient solution, suspension, emulsion, or solid form may also be formulated, i.e., include a buffer, a surfactant, or an excipient which
20 serves to stabilize the active ingredient during drying, e.g., trehalose, ammonium sulfate, 2-hydroxy propyl β -cyclodextrin, sucrose, or other protein-stabilizing sugars or excipients.

A suspension of protein loaded microparticles
25 prepared in accordance with the present invention is preferably administered by injection intraperitoneally, subcutaneously, or intramuscularly. However, it would be clear to one skilled in the art that other routes of delivery could also be effectively utilized using the
30 compositions of the present invention.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof. Example 1

-19-

describes a method for preparing protein loaded microparticles using spray drying for the microparticle preparation. NESP (in the form of a spray dried protein powder) is used as an example protein. Example 2

5 describes various characterization experiments performed on the NESP-containing microparticles of Example 1. Example 3 demonstrates the ability of NESP-containing microparticles of Example 1 to provide for sustained release of NESP *in vivo*. Example 4 describes a novel

10 method for preparing polymeric microparticles wherein a cosolvent is utilized to effect more rapid and effective removal of residual solvent. Example 5 describes a method for preparing NESP-containing microparticles using spray freezing for the microparticle preparation

15 step. Example 6 describes various characterization experiments performed on the NESP-containing microparticles of Example 5. Example 7 demonstrates the ability of NESP-containing microparticles of Example 5 to provide for sustained release of NESP *in vivo*.

20 Example 8 describes the double emulsion/solvent extraction and evaporation method for preparing PLGA microparticles containing NESP, and demonstrates the ability to provide for sustained release of NESP *in vivo*. Example 9 describes the preparation of leptin-

25 containing microparticles using the process of Example 1 and demonstrates the ability of the leptin-containing microparticles to provide for sustained release of leptin *in vivo*. Example 10 describes a method for preparing protein loaded microparticles using spray

30 drying for the microparticle preparation. Example 11 describes various characterization experiments performed on the NESP-containing microparticles of Example 10. Example 12 demonstrates the ability of NESP-containing microparticles of Example 10 to provide for sustained

35 release of NESP *in vivo*. Example 13 demonstrates the

-20-

ability of NESP-containing microparticles of Example 10 to provide for sustained release of NESP in vivo.

EXAMPLE 1

5

This example describes a method for preparing protein loaded microparticles; specifically, the preparation of poly(D,L-lactide-co-glycolide) microspheres containing NESP, using spray drying for the microparticle preparation step.

NESP was formulated at 46% NESP, 29% sodium phosphate salts, 25% trehalose (w/w/w) and spray dried on a lab scale spray dryer (BUCHI 190) using the following conditions: feed rate 2.0 ml/min, atomization 500 NL/hour, inlet temperature 135°C, outlet temperature 99°C, drying gas flow rate 800 SLPM. The protein powder was collected and characterized as described in Example 2 below.

The protein powder was added to a PLGA in dichloromethane (inherent viscosity 0.18, 11 kD) polymer solution (40% w/v) and the resulting suspension was spray dried on a pilot plant scale spray dryer (Niro Mobile Minor™) using the following conditions: feed rate 50 ml/min, atomization flow rate (two-fluid nozzle) 3.0 kg/hr, inlet gas temperature 50°C, outlet temperature 30°C, drying gas flow rate 93 kg/hr. The resulting NESP-containing microparticles (0.53% NESP) were then collected and characterized after sieving (125 µm mesh size).

NESP-containing microparticles of various PLGA composition were produced using this process. The PLGA compositions differed in the copolymer ratio of lactide:glycolide from 50:50 to 100% lactide. The PLGA polymers used had free acid polymer chain end groups.

35

EXAMPLE 2

This example describes various characterization experiments performed on the spray dried NESP/trehalose protein powder and the NESP-containing microparticles described in Example 1.

The NESP/trehalose protein powder was characterized by size exclusion chromatography under native conditions; monomer percent was unchanged post spray drying (>99.8%) (See Figure 2). No additional aggregate were observed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), using silver stain. The protein powder was analyzed by reverse phase high performance liquid chromatography (RP-HPLC); no change from the unprocessed NESP was observed. The protein powder was also characterized by an HPLC glycoform assay and isoelectric focusing gel electrophoresis (IEF) and IEF western blot; no change in glycoform distribution was observed. Radioimmunoassay of the reconstituted powder demonstrated full antibody recognition, and tryptic mapping demonstrated no change in oxidation from the unprocessed material. The particle size distribution of the protein powder was determined by Fraunhofer diffraction to have mean volume distribution of 4.7 μm .

The particle size of the NESP-containing microparticles was $58 \pm 8 \mu\text{m}$ for the mean of the volume distribution determined by Fraunhofer diffraction, averaged over the 5 lots. The post-encapsulation integrity of NESP within these microparticles was assessed by extracting the protein and analyzing the extracts via anion exchange and size exclusion HPLC under native conditions. To extract the NESP from the microparticles, approximately 20 mg of microparticles were placed in a tube with 1 mL acetonitrile. The

-22-

samples were vortexed for 10-20 seconds and centrifuged at 14,000 rpm for 2 minutes at 4°C to pellet the protein and excipients. The supernatant was removed and the pellet resuspended in 1 mL acetonitrile. The above steps were performed three more times. After final supernatant removal, samples were dried in a vacuum oven for 2-3 hours at room temperature. The NESP pellet was reconstituted in 20mM sodium phosphate, pH 6.0, with or without 0.005% Tween 80. After gentle flicking of the tube the sample was incubated at room temperature for 2 hours to achieve full dissolution. Protein was quantitated and integrity determined by anion exchange in series with size exclusion HPLC. Protein recovery was quantitative (>99%) and protein integrity was $98.2 \pm 1.0\%$ monomeric by size exclusion HPLC averaged over the 5 lots, each lot characterized in triplicate (See Figure 2). The extract from the 75% lactide formulation was additionally characterized by radioimmunoassay, capillary electrophoresis, and peptide mapping. The RIA resulted in protein recoveries consistent with the SEC results, demonstrating antibody recognizable protein. Capillary electrophoresis confirmed that the glycoform distribution was unchanged from the unprocessed NESP. The extent of oxidation as determined from peptide mapping was equivalent to the unprocessed NESP.

EXAMPLE 3

This example demonstrates the ability of NESP-containing microparticles prepared as described in Example 1 to provide for sustained release of NESP in vivo.

NESP-containing microparticles (360 $\mu\text{g/kg}$ NESP peptide dose) were injected subcutaneously at the nape of the neck of Male Sprague Dawley rats (385 ± 14 g). A

-23-

subcutaneous, single injection bolus of NESP in a dose equivalent to the microparticles was included as a control. Blood samples were taken from the tail vein at times post injection up to 8 weeks. The NESP

5 concentration in the rat serum was determined by ELISA through 2 and 4 weeks. Whole blood was analyzed for hematocrit, hemoglobin, and reticulocyte counts.

NESP serum levels from a single injection of NESP-containing microparticles fabricated from 50:50
10 lactide:glycolide polymer were > 1 ng/mL for 15 days for all three lots studied (See Figure 3). The NESP alone given as a single bolus had elevated serum levels for 11 days (See Figure 3). The NESP-containing microparticles elevated the hemoglobin and hematocrit above baseline
15 for 25 and 28 days, respectively (See Figure 4). The NESP bolus had elevated hemoglobin and hematocrit for 25 days (See Figure 4).

NESP serum levels from a single injection of NESP-containing microparticles fabricated from 75:25
20 lactide:glycolide polymer were > 1 ng/mL for 20 days (See Figure 3). The NESP-containing microparticles elevated the hemoglobin and hematocrit above baseline for > 40 days (See Figure 4).

NESP serum levels from a single injection of
25 NESP-containing microparticles fabricated using a solution mixture of 50:50 and 100% lactide polymers to create an overall average 75% lactide polymer were > 1 ng/mL for 18 days (See Figure 3). The NESP-containing microparticles elevated the hemoglobin and hematocrit
30 above baseline for 35 days (See Figure 4).

EXAMPLE 4

This example describes the novel method for
35 preparing microparticles wherein a cosolvent is utilized

-24-

to effect more rapid and effective removal of residual solvent; specifically, the preparation of poly(D,L-lactide-co-glycolide) microspheres utilizing an ethanol/methylene chloride cosolvent mixture.

5 Two batches of 50:50 PLGA (11 kD) microparticles were produced by spray drying. For batch 1, pure methylene chloride was used to dissolve a mass of PLGA equal to 26% of the solvent mass. For batch 2, a cosolvent of methylene chloride (86. 4% by mass) and
10 ethanol (13.6% by mass) was used to dissolve a mass of PLGA equal to 26% of the cosolvent mass). The resulting solutions were spray dried on a pilot plant scale spray dryer (Niro Mobile Minor™) using the following conditions: feed rate 50± 5 ml/min, atomization flow
15 rate (two-fluid nozzle) 60 SLPM, inlet gas temperature 55°C, outlet temperature 33-36 °C, drying gas flow rate 2.1 lbs/min. The resulting microparticles were collected and characterized.

 The residual solvent concentration in Batch 1
20 following spray drying was 18550 ppm methylene chloride. The residual solvent concentrations in Batch 2 following spray drying were 6190 ppm methylene chloride, 3330 ppm ethanol. Secondary drying was performed on both batches as follows: the microparticles were placed in a 1.5"
25 diameter dryer on a retaining screen. The dryer was sealed and nitrogen was flowed through the bed at 4.4 L/min. The dryer was submerged in a heating bath for temperature control. After Batch 1 was dried for 73 hours, starting at 20°C and ending at 41°C, the residual
30 methylene chloride level was 750 ppm. Batch 2 was dried for 40 hours, starting at 18°C and ending at 30°C. These microparticles reached residual solvent levels of 487 ppm methylene chloride and 455 ppm ethanol.

 Use of the cosolvent thus decreases the amount
35 of drying time necessary and improves overall residual

-25-

solvents levels in the final microparticles, thus making processes utilizing such solvents more commercially practicable.

5

EXAMPLE 5

This example describes a method for preparing protein loaded microparticles; specifically, the preparation of poly(D,L-lactide-co-glycolide) microspheres containing NESP, using spray freezing for the microparticle preparation step.

Two formulations of NESP (trehalose formulation from Example 1, and an ammonium sulfate formulation) were spray dried on a lab scale spray dryer using conditions described in Example 1. The ammonium sulfate formulation for NESP was: 11% NESP, 10% phosphate salts, 79% ammonium sulfate (w/w/w).

Microparticles containing the NESP/trehalose protein powder were prepared from unblocked PLGA obtained from either Alkermes/Medisorb Wilmington, Ohio, or Boehringer Ingelheim Chemicals, Inc., Montvale, N.J. or blocked PLGA from Boehringer Ingelheim. The polymer inherent viscosities ranged from 0.14 dL/g to 0.5 dL/g (11 - 47 kD molecular weight), and the lactide contents were from 50% to 100%. NESP/ammonium sulfate protein powder was encapsulated in unblocked PLGA (50:50) with an inherent viscosity of 0.18 dL/g (11 kD).

The method described by Gombotz et al. (U.S. Pat. No. 5,019,400) was used to encapsulate the spray dried NESP powder in PLGA. The protein powder was added to a polymer solution of PLGA in dichloromethane (5 - 20% w/v) to achieve a protein solids content in the microparticles ranging from 1 - 5% (w/w). A container of ethanol was frozen by immersion in liquid nitrogen, and overlaid with a layer of liquid nitrogen prior to

-26-

the spray freezing step. The suspension of protein powder in polymer solution was pumped via a syringe pump to an ultrasonic nozzle placed above the frozen bath of ethanol. The suspension was atomized into droplets that
5 froze upon contact with the liquid nitrogen and settled onto the surface of the frozen ethanol, forming microparticles. The frozen bath was transferred to -80 °C for 72 hours to allow the ethanol to melt and extract the polymer solvent from the microparticles. The
10 resulting slurry of microparticles in ethanol was cold-filtered (0.65 μ m PTFE) and the collected microparticles were lyophilized. Following lyophilization the microparticles (0.53% NESP peptide content) were sieved (125 μ m mesh size) prior to characterization.

15

EXAMPLE 6

This example describes various characterization experiments performed on the spray
20 freeze NESP-containing microparticles described in Example 5.

The NESP protein powders were characterized as described in Example 2, with the results for the NESP/trehalose already presented there. NESP/ammonium
25 sulfate (AS) was characterized by size exclusion chromatography under native conditions; monomer percent was significantly decreased post spray drying, by 2.5%. This was a dimer confirmed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and
30 demonstrated to be non-reducible, using silver stain. The particle size distribution of the NESP/AS protein powder was determined by Fraunhofer diffraction to have mean volume distribution of 4.3 μ m.

The particle size of the NESP-containing
35 microparticles fabricated by spray freezing for 28

-27-

unique lots of various formulations ranged from 20 to 45 μm for the mean of the volume distribution. The residual methylene chloride levels were all < 500 ppm for the 10 lots evaluated. The post-encapsulation integrity of NESP within these microparticles was assessed by extracting the protein and analyzing the extracts as described in Example 2. Protein recovery was quantitative and integrity (monomer %) was $97.2 \pm 2\%$, each lot characterized in at least duplicate. The extracts from 9 select formulations were additionally characterized by a radioimmunoassay, capillary electrophoresis, and peptide mapping. The RIA resulted in protein recoveries consistent with the SEC results, demonstrating antibody recognizable protein. Capillary electrophoresis confirmed that the glycoform distribution was unchanged from the unprocessed NESP. The extent of oxidation of the protein determined from the peptide mapping ranged from 7 - 12%, unprocessed material is typically $8 \pm 2\%$.

20

EXAMPLE 7

This example demonstrates the ability of NESP-containing microparticles prepared in Example 5 to provide for sustained release of NESP in vivo.

Male Sprague Dawley rats were treated as described in Example 3 with different formulations of NESP-containing microparticles fabricated by the spray freeze process as described in Example 6. Serum NESP concentration and whole blood analyses were performed through 4-6 weeks as described in Example 3.

Figure 5 shows the results obtained for the NESP serum levels using various copolymer formulations of Example 5. All of the copolymer formulations demonstrated a burst phase followed by a phase of zero-

-28-

order release, and then a drop to below the assay quantitation limit. Increasing the lactide content decreased the NESP serum level through the zero order phase by over 100-fold. Increasing the inherent viscosity (molecular weight) likewise decreased this NESP serum level, but only by 4-fold (See Figure 6). Both increasing the inherent viscosity and increasing the lactide content, whether by copolymer composition or polymer solution mixture, increased the duration of quantifiable serum NESP concentrations from 18 days to as long as 35 days.

The pharmacodynamic effect, as measured by elevated hematocrit above baseline levels, paralleled the trends observed with the serum NESP concentration. Preliminary data on NESP serum concentrations and the elevation of reticulocytes counts suggest an efficacious serum level in rats to be near 0.4 ng/mL for treatment with NESP microparticles.

In a mouse pharmacodynamic study, male BDF1 mice (body weight 22 g) were treated with NESP/PLGA microparticles (50:50, inherent viscosity 0.4 dL/g), in a single, subcutaneous bolus injection into the nape of the neck, at NESP doses of 6, 30 and 100 μ g/kg. NESP solution test groups were dosed in a single, subcutaneous bolus injection into the nape of the neck, at NESP doses of 100, 1,000 and 10,000 μ g/kg. The study design was such that blood was collected every 2-4 days for whole blood analysis over a 35 day period, but any individual animal was not bled more than twice in a 7 day period.

A dose response of the microparticle treated groups was observed. Hemoglobin levels were elevated above baseline for > 4weeks for the microparticle dose of 100 μ g/kg. The duration of the effect after treatment with NESP solution bolus was only equivalent

-29-

to that observed with the microparticles when given at 100-fold greater dose than the microparticles (See Figure 7).

5

EXAMPLE 8

This example describes the double emulsion/solvent extraction and evaporation method for preparing PLGA microparticles containing NESP, and demonstrates the ability to provide for sustained release of NESP in vivo.

Two aqueous formulations of NESP were prepared: formulation 1 = 5.5 mg/mL NESP (peptide concentration) formulated with 20 mM sodium phosphate, pH 6.0; formulation 2 = 5.2 mg/mL NESP (peptide concentration) formulated with 20 mM sodium phosphate, 105 mg/mL 2-hydroxypropyl β -cyclodextrin, pH 6.0.

Microparticles containing the above NESP formulations were prepared from unblocked PLGA (50:50, inherent viscosity 0.2, 11 kD) obtained from Boehringer Ingelheim Chemicals, Inc., Montvale, N.J. The double emulsion followed by solvent extraction and evaporation method was used as described below.

Approximately 2 g of polymer was dissolved in 6 mL of dichloromethane and homogenized at 25 krpm in an 18 x 150 mm glass test tube on ice. Protein solution was added (1.0 mL) during homogenization which was continued for an additional 30 seconds on ice to form the primary emulsion. For the secondary emulsion, 40 mLs of the aqueous outer phase (18 mM sodium phosphate, 0.5% polyvinyl alcohol, pH 6.0) in a 100 mL beaker of 4.5 cm inner diameter, was prechilled to 15 C in a water bath. A 1" Rushton impeller blade was immersed in the chilled outer phase and mixing was initiated at 1480 rpm. The primary emulsion was added quickly to form the

-30-

secondary emulsion, and mixing was continued at the same speed for a total of 40 minutes.

The cured emulsion was washed with water twice by transferring the solution to two 50 mL tubes, centrifuging at 500 g-force for 30 seconds, decanting to a volume of 10 mLs, and repeating. The final suspension was transferred into vials and lyophilized. The final dry powder was sieved (180 μ m mesh size) prior to characterization by methods described in Example 2.

The process provided quantitative encapsulation efficiency, poor product yield (50%); large particle size (120 μ m), and acceptable residual methylene chloride levels (near 500 ppm). The post-encapsulation integrity of NESP within these microparticles was assessed by extracting the protein and analyzing the extracts as described in Example 2. Protein recovery was quantitative and integrity (monomer %) was $97.5\% \pm 0.01\%$ for formulation (1), and $96.7 \pm 0.3\%$ for formulation (2); each lot was characterized in triplicate.

Male Sprague Dawley rats were treated as described in Example 3 with the two formulations of NESP-containing microparticles fabricated by the double emulsion process. Serum NESP levels demonstrated a burst phase followed by a phase of zero-order release, and then a drop to below the assay quantitation limit after 18 days for formulation 1 and after 22 days for formulation 2. The pharmacodynamic effect as measured by hemoglobin elevated above baseline, lasted for 25 and >28 days (end of study) for formulations (1) and (2), respectively (See Figure 8).

-31-

EXAMPLE 9

This example describes the preparation of microparticles containing leptin and demonstrates the ability of leptin-containing microparticles to provide for sustained release of leptin *in vivo*.

Leptin-containing microparticles were prepared using the procedure described in Example 1, and then characterized as described in Example 2. It was determined that protein recovery was >95% and the integrity was >98%. The process provided high encapsulation efficiency (85-95%), good product yield (75-85%), low burst (<15%), acceptable particle size (~35 μ m), and acceptable residual solvent levels. It was further demonstrated that the formulation exhibited good storage stability.

"*In vivo*" bioactivity of leptin-containing microparticles were evaluated in normal rats. A total leptin dose of 50 mg/kg (corresponding to ~150 mg microparticles/kg) were administered as a single injection at day 0. In addition, the following control groups were included: daily leptin bolus (5 mg/kg/day x 10 days); dose dump control (50 mg/kg at day 0); placebo microparticles administered at day); and daily placebo injection control. Animals were weighed daily, and serum samples collected periodically to assess the serum leptin concentration.

Serum leptin levels are shown in Figure 9. The leptin concentration in the serum remains above baseline for approximately 5 days. Weight loss is presented in Figure 10. % body weight loss relative to buffer control was determined for a 30 day period. Daily injection of a leptin solution resulted in 4-6% weight loss relative to placebo controls. A single injection of leptin-containing microparticles resulted

-32-

in 9-10% weight loss relative to placebo microparticles, and resulted in sustained weight loss in rats for a 25 day period.

After day 10, some animals were sacrificed for
5 histological examination of the injection site.
Histological examination of the injection site revealed a localized minimal to mild inflammatory reaction, which was fully reversible with biodegradation of the microparticles over time.

10

Example 10

This example describes a method for preparing protein loaded microparticles; specifically, the
15 preparation of poly(D,L-lactide-co-glycolide)
microspheres containing NESP, using spray drying for the microparticle preparation step.

NESP was formulated at 46% NESP, 29% sodium phosphate salts, 25% trehalose (w/w/w) and spray dried
20 on a pilot plant scale spray dryer (Niro Mobile Minor™) using the following conditions: feed rate 8.0 ml/min, atomization gas 0.33 lbs/min (two-fluid nozzle), inlet temperature 200°C, outlet temperature 100°C, drying gas flow rate 2.0 lbs/min. The protein powder was collected
25 and characterized as described in Example 11 below.

The protein powder was added to a PLGA (high molecular weight, (inherent viscosity 0.49 dL/g), 50% lactide) in dichloromethane/ethanol polymer solution (10% w/v) and the resulting suspension was spray dried
30 on a pilot plant scale spray dryer (Niro Mobile Minor™) using the following conditions: feed rate 12 ml/min, atomization (ultrasonic nozzle) 1.3 watts, inlet gas temperature 55°C, outlet temperature 28°C, drying gas flow rate 1.2 lbs/min. The resulting NESP-containing

-33-

microparticles (0.53% NESP) were then collected and secondary drying was performed as described in Example 4 to reduce residual solvent concentrations to less than 1000 ppm. The resulting microparticles were
5 characterized after sieving (125 μm mesh size).

Example 11

This example describes various
10 characterization experiments performed on the spray dried NESP/trehalose protein powder and the NESP-containing microparticles described in Example 10.

The NESP/trehalose protein powder was characterized by size exclusion chromatography under
15 native conditions; monomer percent was 99.8% post spray drying (100% for un-processed material). No additional aggregate was observed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), using silver stain. The protein powder was analyzed by reverse phase high
20 performance liquid chromatography (RP-HPLC); no change from the unprocessed NESP was observed. The protein powder was also characterized by an HPLC glycoform assay and isoelectric focusing gel electrophoresis (IEF) no
25 change in glycoform distribution was observed. The particle size distribution of the protein powder was determined by Fraunhofer diffraction to have a mean size of 2.5 μm (volume distribution)

The NESP-containing microparticles were determined by Fraunhofer diffraction to have a mean
30 size of $45 \pm 1 \mu\text{m}$ (volume distribution). Residual solvent concentrations of dichloromethane and ethanol were determined by head space gas chromatography to be 638 ppm and <100 ppm, respectively.

The post-encapsulation integrity of NESP
35 within these microparticles was assessed by extracting

-34-

the protein as described in Example 2 and analyzing the extracts via anion exchange and size exclusion HPLC under native conditions. Protein was quantitated and integrity determined by anion exchange in series with size exclusion HPLC. Protein recovery was quantitative (>99%) and protein integrity was $98.6 \pm 0.3\%$ monomeric by size exclusion HPLC. No additional aggregates were observed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), using silver stain. The NESP microparticle protein extract was analyzed by reverse phase high performance liquid chromatography (RP-HPLC); no change from the NESP protein powder was observed. The NESP microparticle protein extract was also characterized by an HPLC glycoform assay and isoelectric focusing gel electrophoresis (IEF) no change in glycoform distribution was observed.

Example 12

This example demonstrates the ability of NESP-containing microparticles prepared in Example 10 to provide for sustained release of NESP *in vivo*.

Male Sprague Dawley rats were treated as described in Example 3 with NESP-containing microparticles fabricated as described in Example 10. Serum NESP concentration and whole blood analyses were performed through 4-8 weeks as described in Example 3.

NESP serum levels from a single injection of NESP-containing microparticles were > 1 ng/mL for 20 days (See Figure 11). The NESP alone given as a single bolus had elevated serum levels for 11 days (See Figure 11). The NESP-containing microparticles elevated hematocrit above baseline for 34 days (See Figure 12). The NESP bolus had elevated hematocrit for 20 days (See Figure 12).

-35-

Example 13

5 This example demonstrates the ability of NESP-
containing microparticles prepared in Example 10 to
provide for sustained release of NESP *in vivo*.

Male NIHHRNU-M Nude rats were treated as
described in Example 3 with NESP-containing
microparticles fabricated as described in Example 10.
10 Serum NESP concentration and whole blood analyses were
performed through 4-8 weeks as described in Example 3.

NESP serum levels from a single injection of
NESP-containing microparticles were > 1 ng/mL for 21
days (See Figure 13). The NESP alone given as a single
15 bolus had elevated serum levels for less than 11 days
(See Figure 13). The NESP-containing microparticles
elevated hematocrit above baseline for 44 days (See
Figure 14). The NESP bolus had elevated hematocrit for
18 days (See Figure 14).

20

Materials and Methods

The present NESP may be prepared according to
the above incorporated-by-reference PCT Application No.
25 US94/02957.

The present recombinant methionyl-human-OB
protein (leptin) may be prepared according to the above
incorporated-by-reference PCT publication, WO 96/05309
at pages 151-159. For the present working examples, a
30 human OB protein was used which has (as compared to the
amino acid sequence at page 158) a lysine at position 35
instead of an arginine, and an isoleucine at position 74
instead of an isoleucine. Other recombinant human OB
proteins may be prepared according to methods known

-36-

generally in the art of expression of proteins using recombinant DNA technology.

While the present invention has been described
5 in terms of certain preferred embodiments, it is
understood that variations and modifications will occur
to those skilled in the art. Therefore, it is intended
that the appended claims cover all such equivalent
variations which come within the scope of the invention
10 as claimed.

-37-

CLAIMS:

1. A pharmaceutical composition for the sustained-release of an active ingredient comprising a biologically active ingredient contained within polymeric microparticles, said composition prepared by a method comprising:
 - (a) obtaining a dried powder of said active ingredient or formulated active ingredient;
 - (b) preparing a polymer solution comprising a polymer dissolved in a cosolvent mixture;
 - (c) dispersing said dried powder in said polymer solution to produce an active ingredient/polymer mixture;
 - (d) preparing active ingredient-containing microparticles from said mixture;
 - (e) collecting said microparticles; and
 - (f) finishing said microparticles by secondary drying.
2. A composition according to Claim 1 wherein said active ingredient is selected from the group consisting of peptides, small molecules, carbohydrates, nucleic acids, lipids, proteins, and analogs thereof.
3. A composition according to Claim 2 wherein said active ingredient is a protein.
4. A composition according to Claim 3 wherein said protein is NESP or a chemically modified form thereof.
5. A composition according to Claim 4 wherein said NESP has an amino acid sequence as depicted in SEQ ID NO: 2.

-38-

6. A composition according to Claim 3 wherein said protein is leptin or a chemically modified form thereof.

5

7. A composition according to Claim 1 wherein said polymer is selected from the group consisting of poly(lactide)s, poly(glycolide)s, poly(lactic acid)s, poly(glycolic acid)s, polyanhydrides, polyorthoesters, polyetheresters, polycaprolactone, polyesteramides, polyphosphazenes, polyphosphoesters, pseudo-polyamino acids, blends and copolymers thereof.

8. A method for making a sustained-release pharmaceutical composition comprising an active ingredient contained within polymeric microparticles, said method comprising:

- (a) obtaining a dried powder of said active ingredient or formulated active ingredient;
- (b) preparing a polymer solution comprising a polymer dissolved in a cosolvent mixture;
- (c) dispersing said dried powder in said polymer solution to produce an active ingredient/polymer mixture;
- (d) preparing active ingredient-containing microparticles from said mixture;
- (e) collecting said microparticles; and
- (f) finishing said microparticles by secondary drying.

30

9. A method according to Claim 8 wherein said polymer is selected from the group consisting of poly(lactide)s, poly(glycolide)s, poly(lactic acid)s, poly(glycolic acid)s, polyanhydrides, polyorthoesters, polyetheresters, polycaprolactone, polyesteramides,

35

-39-

polyphosphazenes, polyphosphoesters, pseudo-polyamino acids, blends and copolymers thereof.

10. A method according to Claim 8, wherein
5 said cosolvent mixture is comprised of a polymer solvent and a non-solvent.

11. A method according to Claim 10, wherein
said polymer solvent is methylene chloride and wherein
10 said non-solvent is ethanol.

12. A pharmaceutical composition comprising
NESP or formulated NESP contained within biodegradable,
biocompatible polymeric microspheres, said composition
15 providing release of said NESP over a period of at least
one month after administration to a patient.

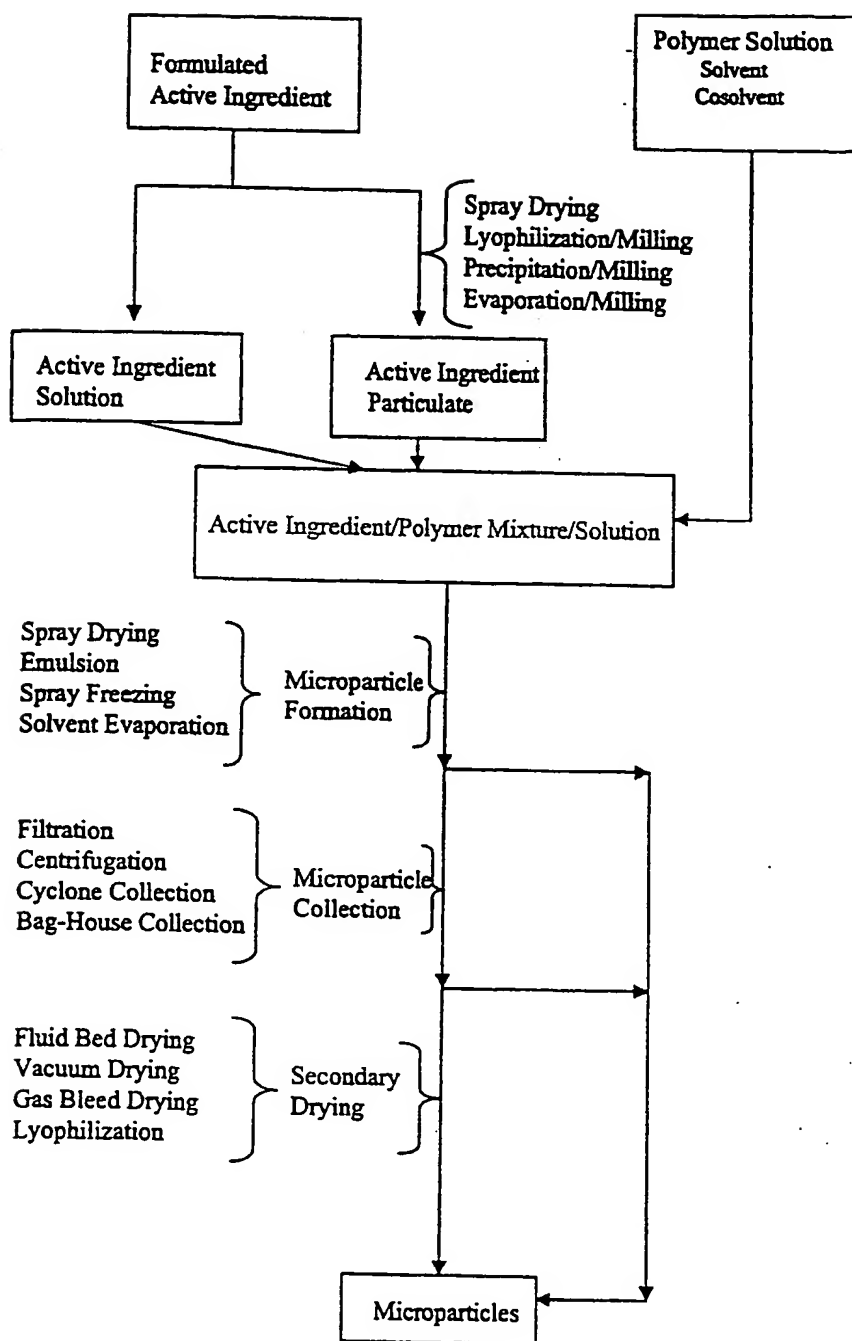


FIGURE 1

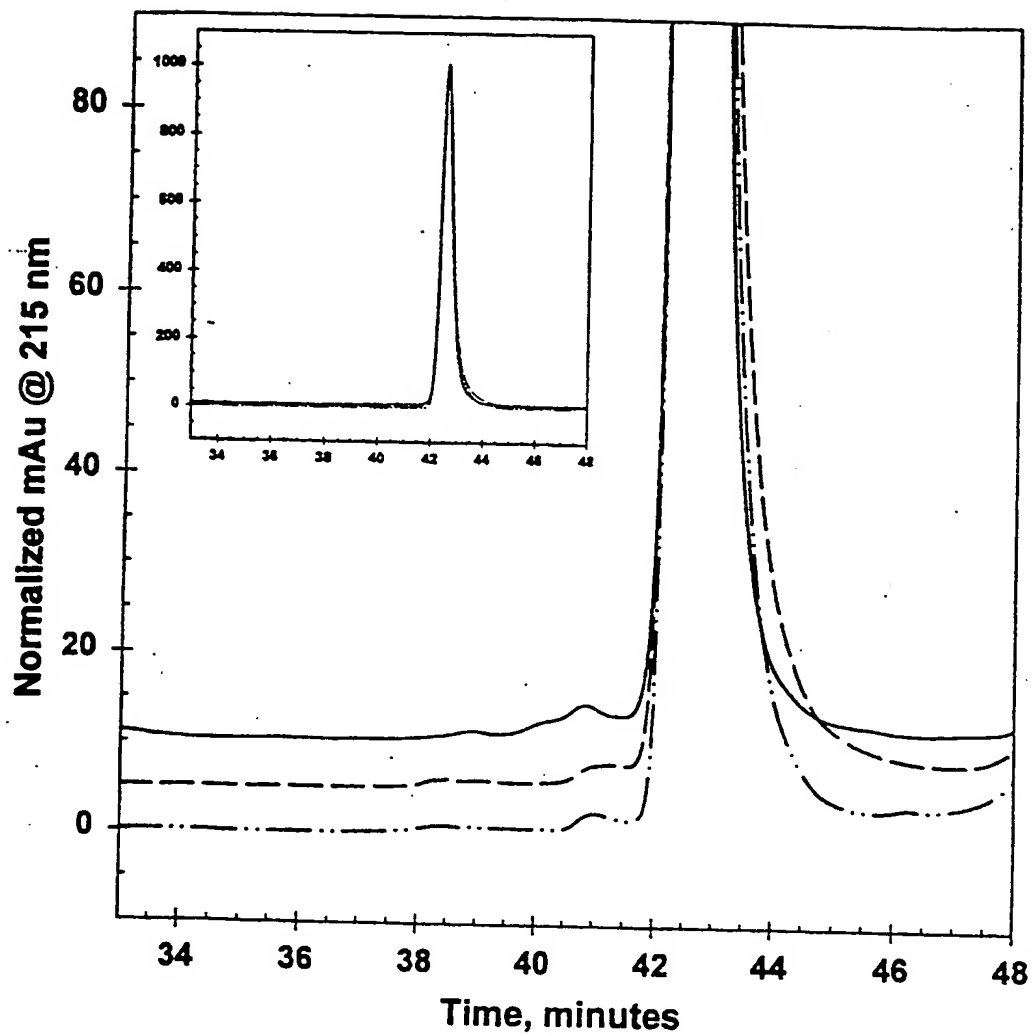


FIGURE 2

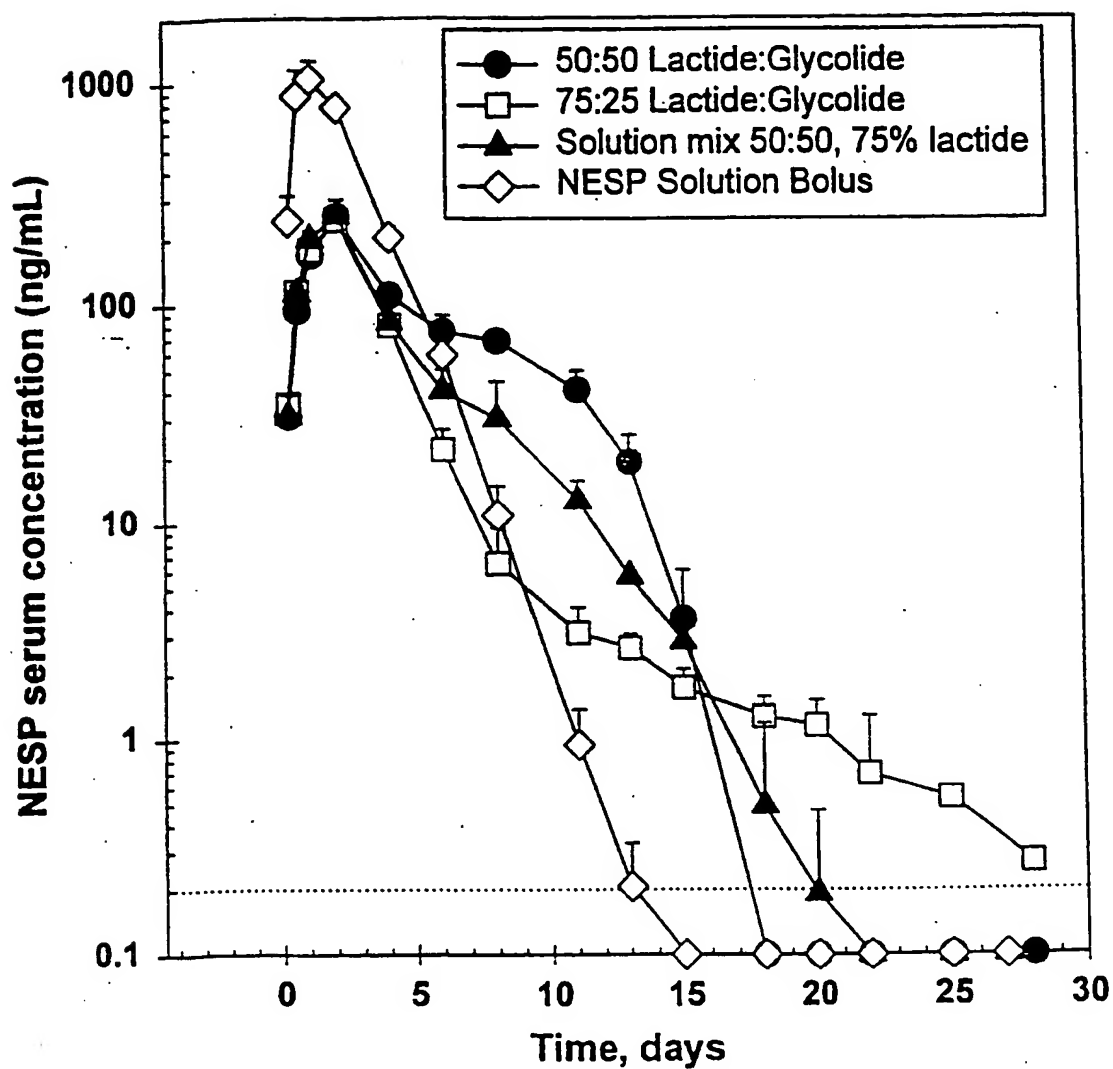


FIGURE 3

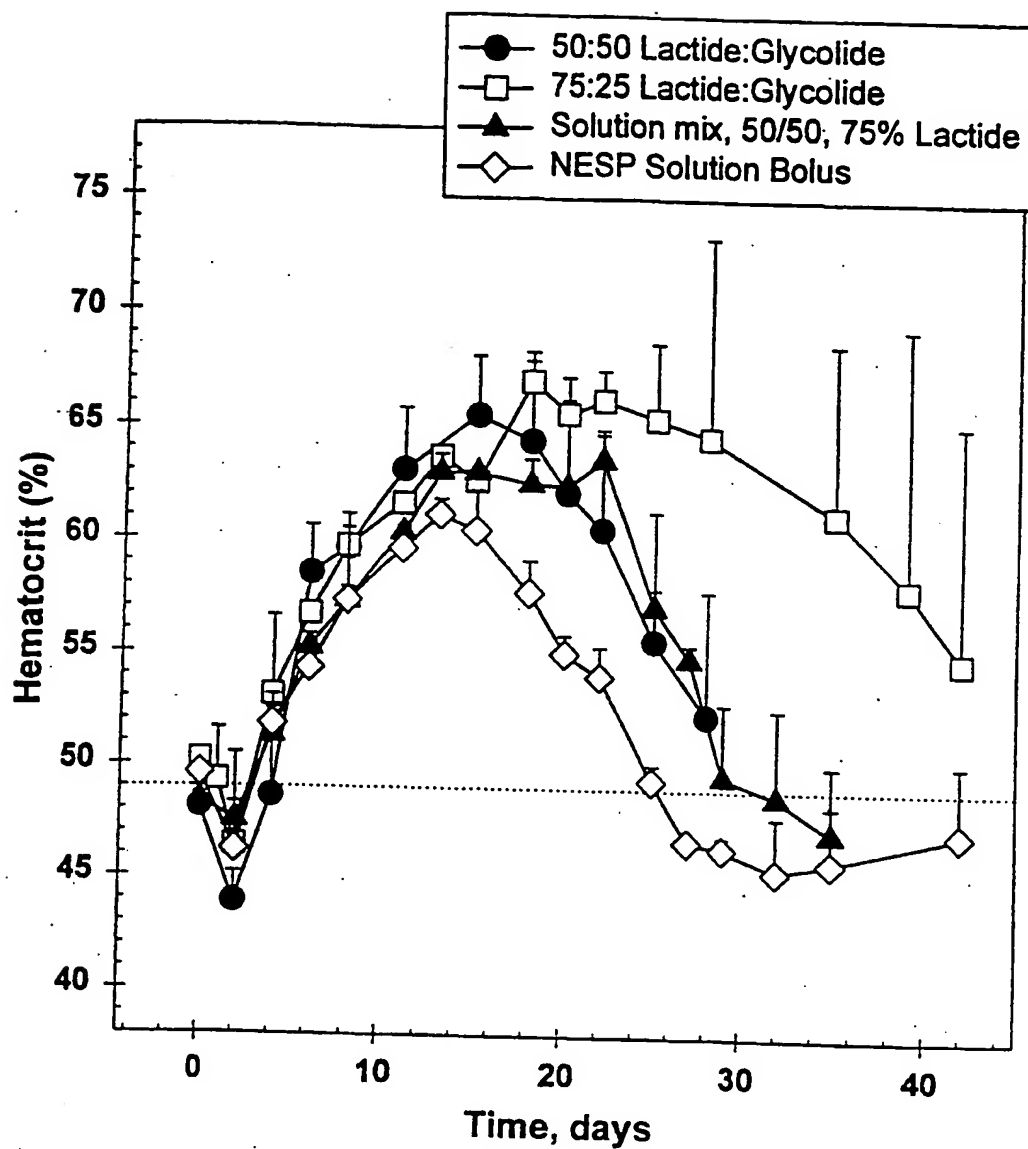


FIGURE 4

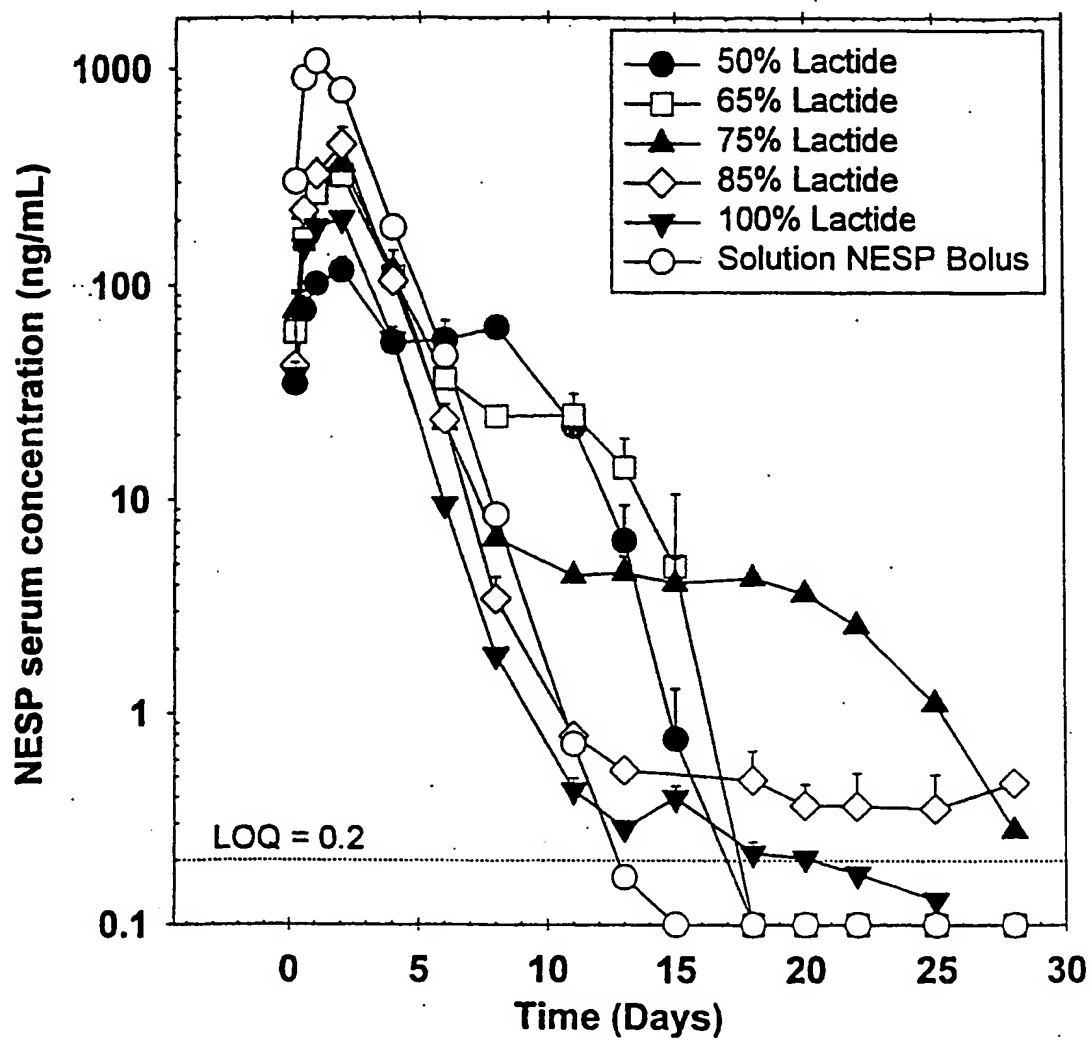


FIGURE 5

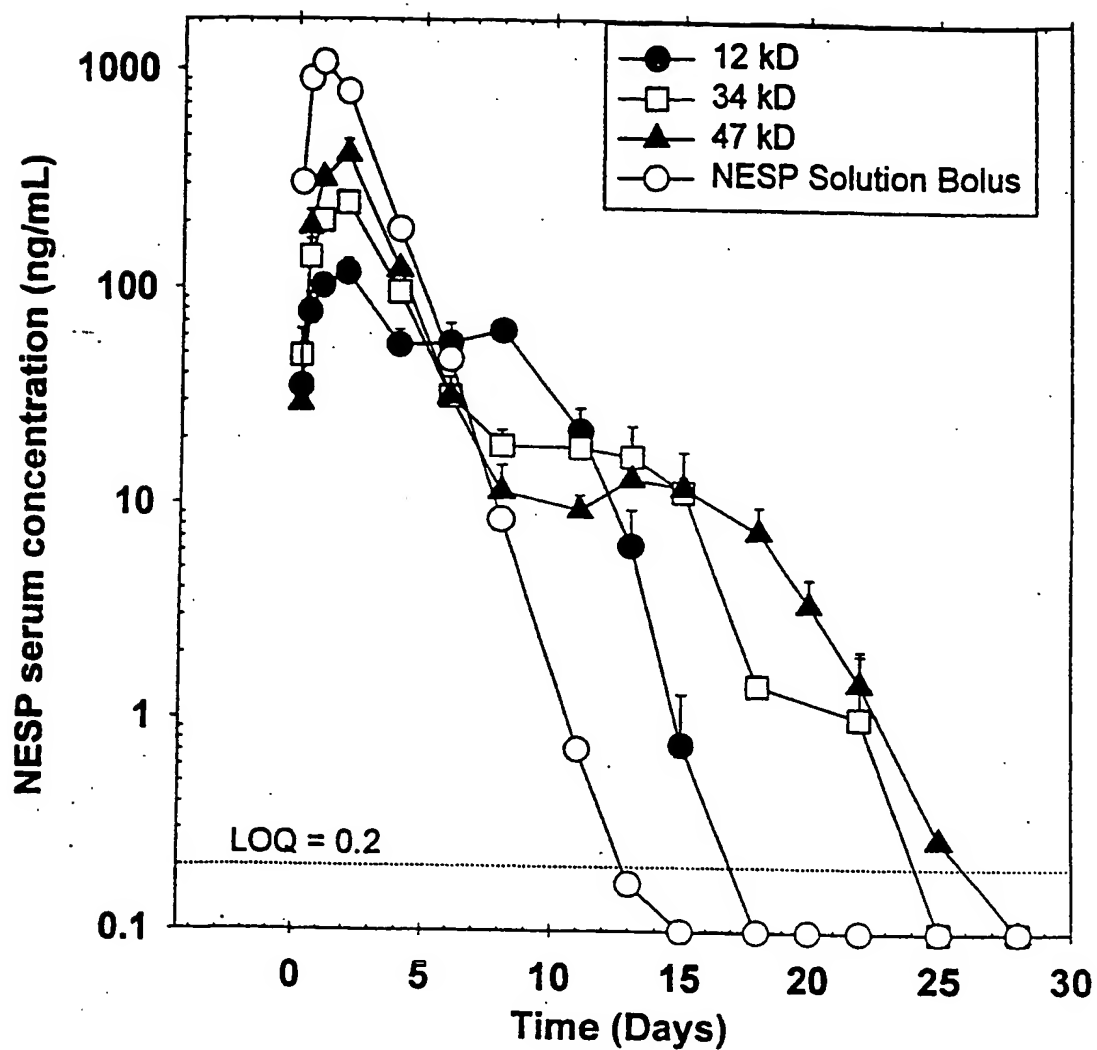


FIGURE 6

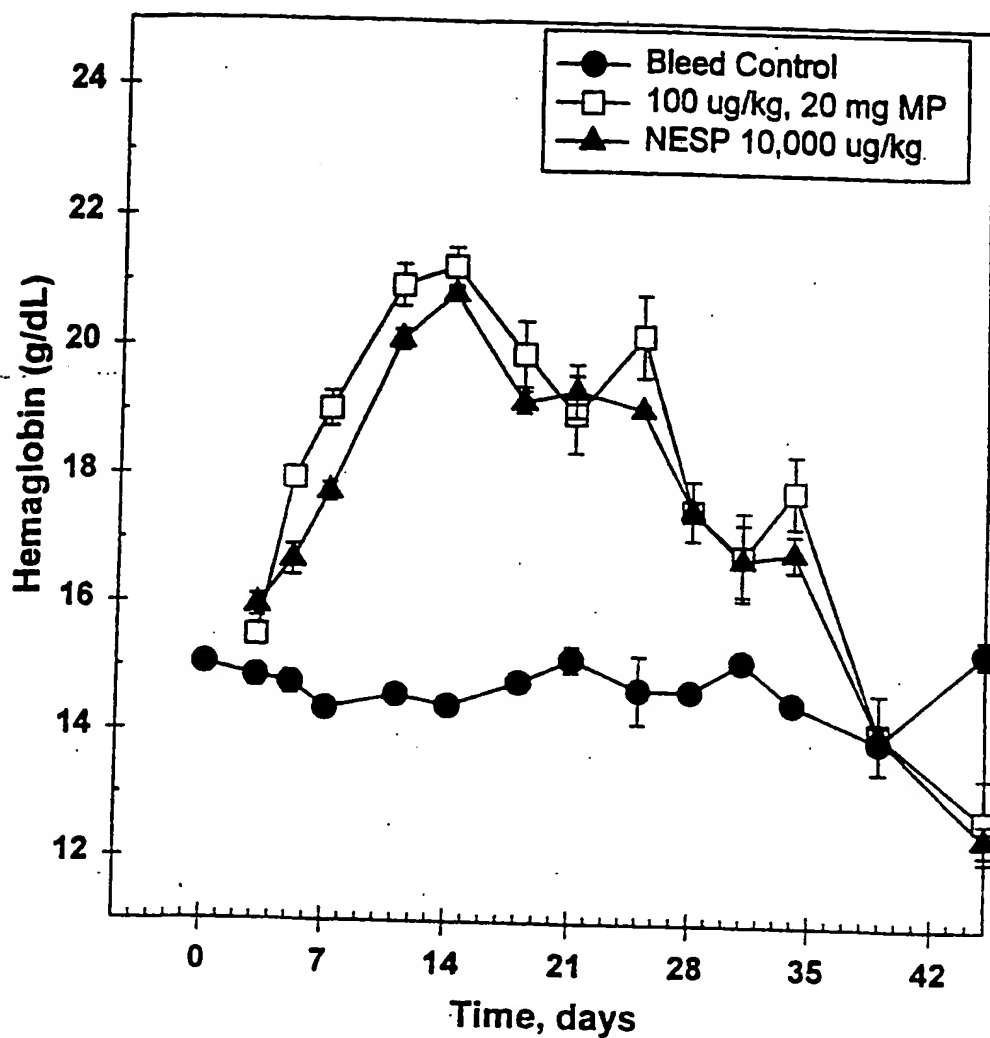


FIGURE 7

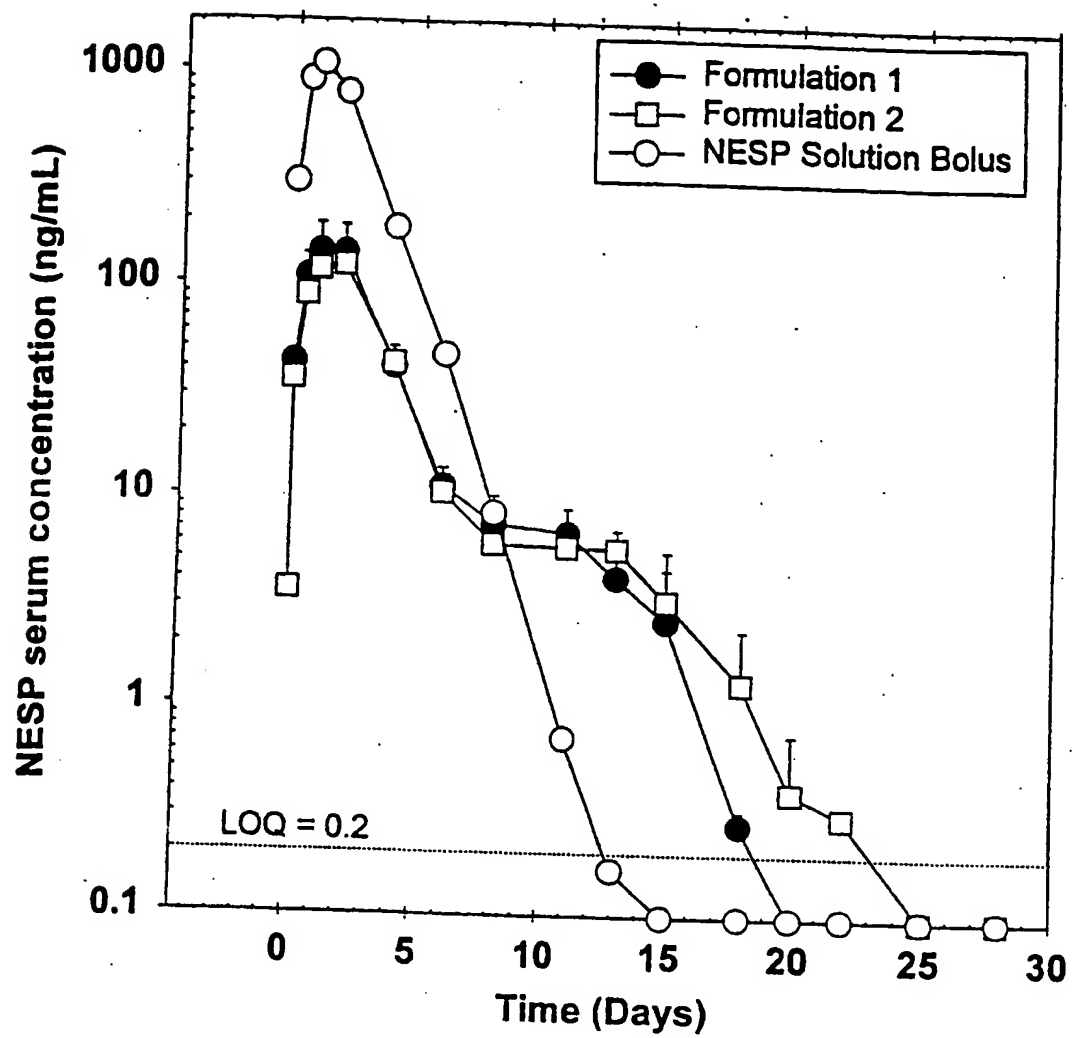


FIGURE 8

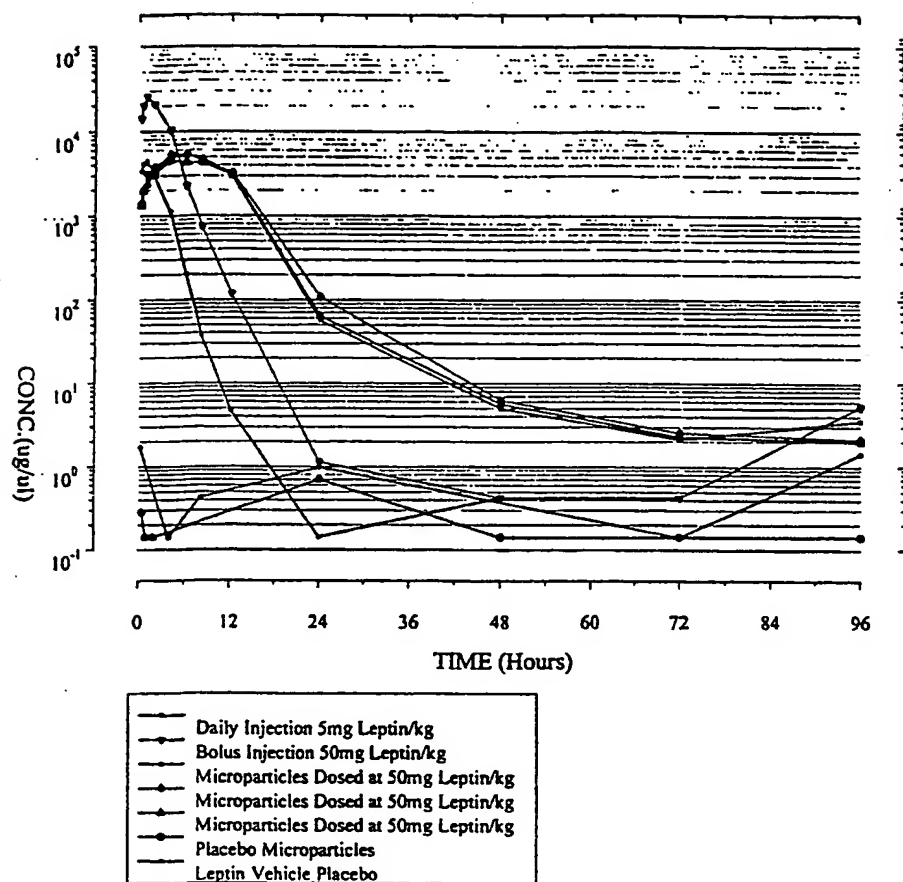


FIGURE 9

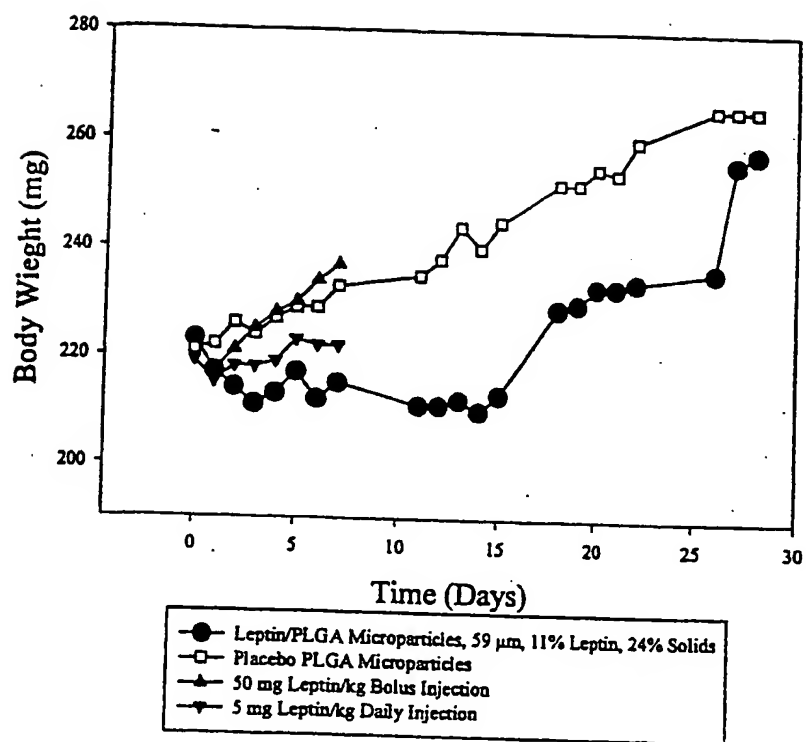


FIGURE 10

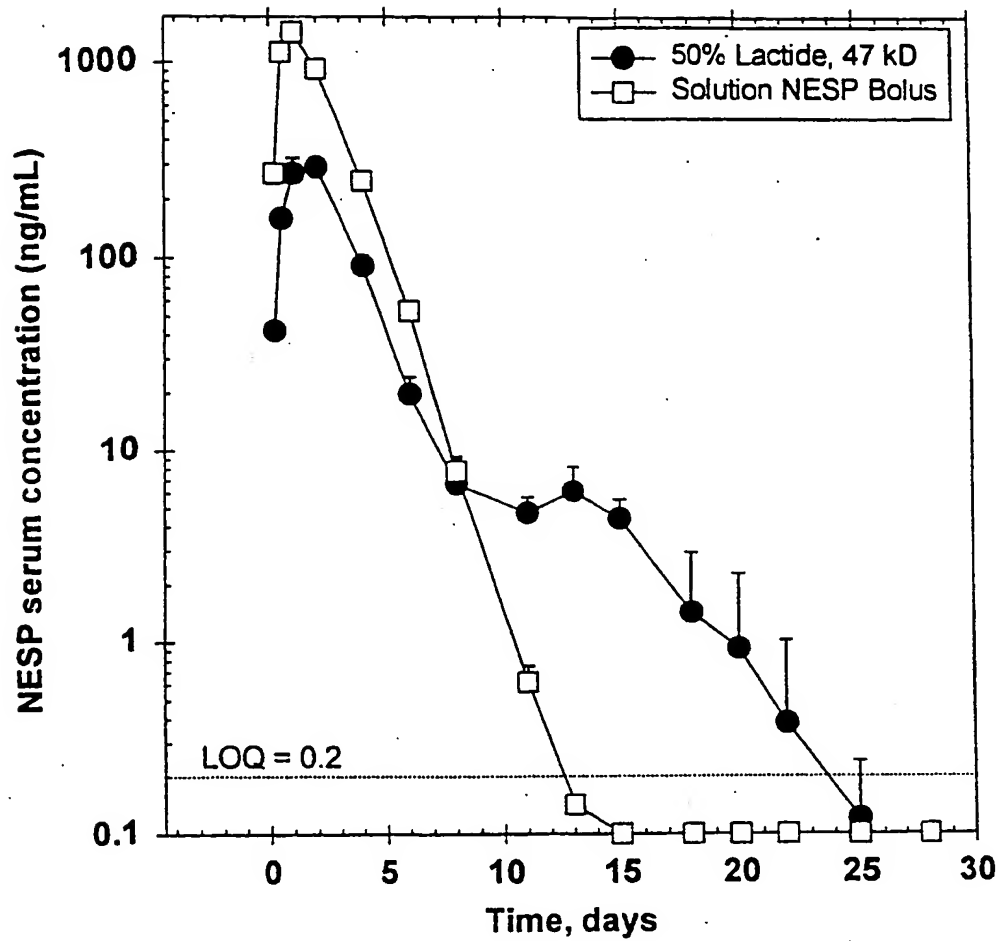


FIGURE 11

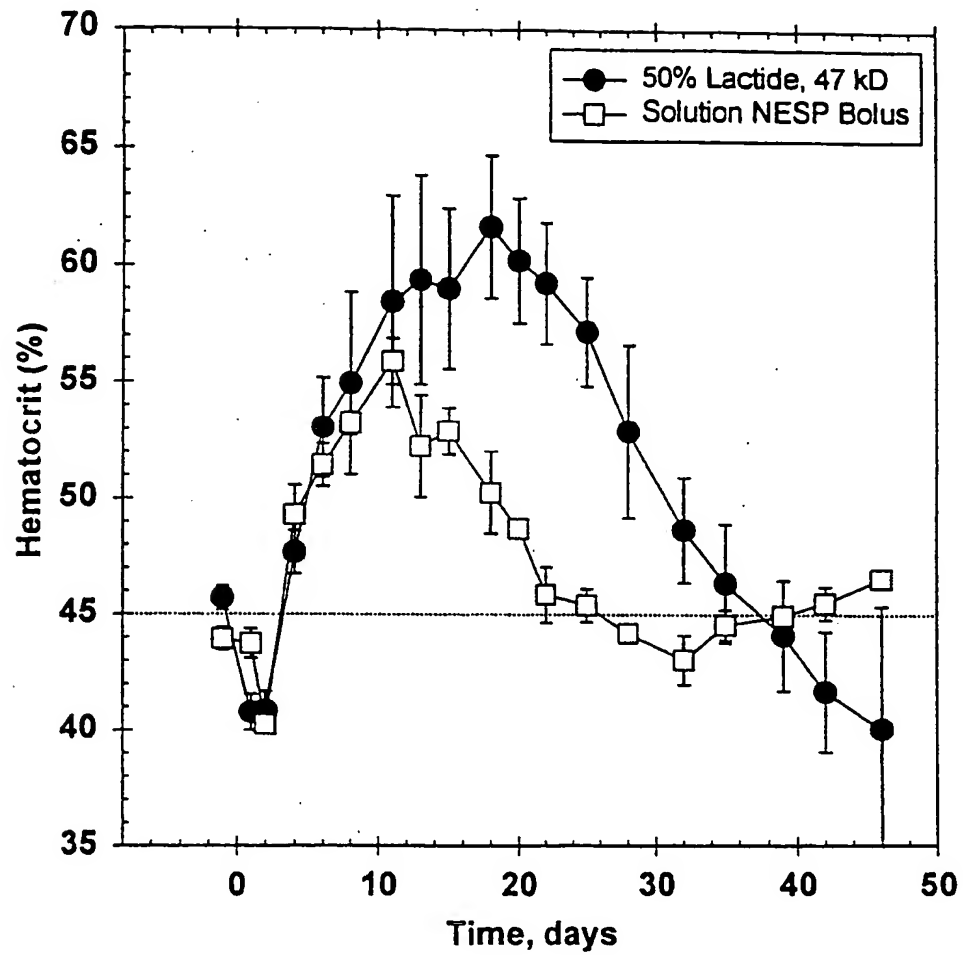


FIGURE 12

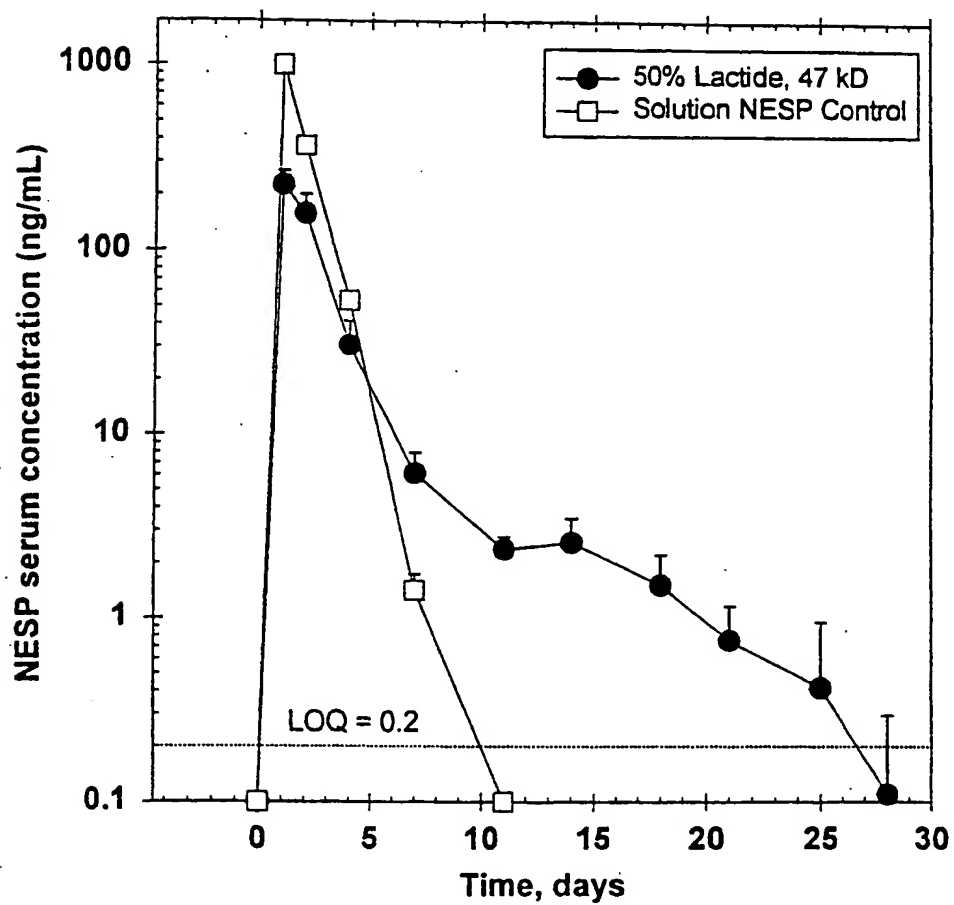


FIGURE 13

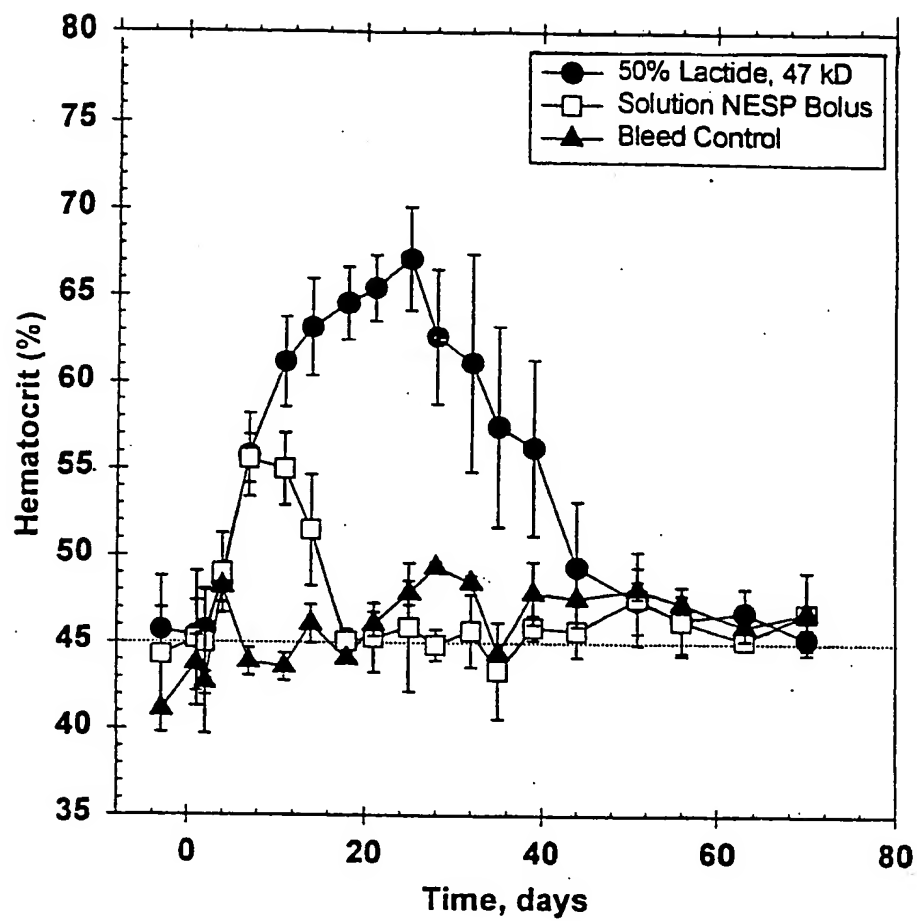


FIGURE 14

SEQUENCE LISTING

<110> AMGEN INC.

<120> BIODEGRADABLE MICROPARTICLES FOR THE SUSTAINED DELIVERY
OF NOVEL ERYTHROPOIETIN STIMULATING PROTEIN

<130> A-626

<140> TO BE ASSIGNED

<141> 1999-10-22

<160> 2

<170> PatentIn Ver. 2.1

<210> 1

<211> 165

<212> PRT

<213> HUMAN

<400> 1

Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp	Ser	Arg	Val	Leu	Glu	Arg	Tyr	Leu
1				5					10					15	

Leu	Glu	Ala	Lys	Glu	Ala	Glu	Asn	Ile	Thr	Thr	Gly	Cys	Ala	Glu	His
		20					25						30		

Cys	Ser	Leu	Asn	Glu	Asn	Ile	Thr	Val	Pro	Asp	Thr	Lys	Val	Asn	Phe
		35				40						45			

Tyr	Ala	Trp	Lys	Arg	Met	Glu	Val	Gly	Gln	Gln	Ala	Val	Glu	Val	Trp
50					55						60				

Gln	Gly	Leu	Ala	Leu	Leu	Ser	Glu	Ala	Val	Leu	Arg	Gly	Gln	Ala	Leu
65				70						75					80

Leu	Val	Asn	Ser	Ser	Gln	Pro	Trp	Glu	Pro	Leu	Gln	Leu	His	Val	Asp
			85					90					95		

Lys	Ala	Val	Ser	Gly	Leu	Arg	Ser	Leu	Thr	Thr	Leu	Leu	Arg	Ala	Leu
		100					105						110		

Gly	Ala	Gln	Lys	Glu	Ala	Ile	Ser	Pro	Pro	Asp	Ala	Ala	Ser	Ala	Ala
		115				120					125				

Pro	Leu	Arg	Thr	Ile	Thr	Ala	Asp	Thr	Phe	Arg	Lys	Leu	Phe	Arg	Val
130					135						140				

Tyr	Ser	Asn	Phe	Leu	Arg	Gly	Lys	Leu	Lys	Leu	Tyr	Thr	Gly	Glu	Ala
145				150					155					160	

Cys	Arg	Thr	Gly	Asp
				165

<210> 2

<211> 165

<212> PRT

<213> HUMAN

<400> 2

Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu
 1 5 10 15

Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Asn Glu Thr
 20 25 30

Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe
 35 40 45

Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp
 50 55 60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu
 65 70 75 80

Leu Val Asn Ser Ser Gln Val Asn Glu Thr Leu Gln Leu His Val Asp
 85 90 95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu
 100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
 115 120 125

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
 130 135 140

Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala
 145 150 155 160

Cys Arg Thr Gly Asp
 165

INTERNATIONAL SEARCH REPORT

Inter national Application No
PCT/US 00/29257

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K9/16 A61K38/18 A61K38/22 C07K14/575 C07K14/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 01331 A (IGARI YASUTAKA ;MISAKI MASAFUMI (JP); OKAMOTO KAYOKO (JP); YAMAGATA) 16 January 1997 (1997-01-16) page 4, paragraph 2 - last paragraph page 13, paragraph 3 - last paragraph page 14, line 25 -page 15, line 10; claims 1,4,6-14; examples 1,7	1-3,7-11
X	WO 95 11009 A (GENENTECH INC) 27 April 1995 (1995-04-27) page 4, line 14 -page 5, line 14; figure 3 page 6, line 10 - line 33 page 7, line 19 -page 8, line 25 page 15, line 26 - last line; claims; table 7	1-3,7-10

-/-

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *A* document member of the same patent family

Date of the actual completion of the international search

9 February 2001

Date of mailing of the international search report

20/02/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Marttin, E

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/29257

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>JEYANTHI R ET AL: "EFFECT OF SOLVENT REMOVAL TECHNIQUE ON THE MATRIX CHARACTERISTICS OF POLYLACTIDE/GLYCOLIDE MICROSPHERES FOR PEPTIDE DELIVERY" JOURNAL OF CONTROLLED RELEASE, NL, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 38, no. 2/03, 1 February 1996 (1996-02-01), pages 235-244, XP000558712 ISSN: 0168-3659 page 236, left-hand column, paragraph 3 -page 237, left-hand column, last paragraph page 237, right-hand column, last paragraph -page 237, right-hand column, last paragraph.</p>	1-3,7-11
X	<p>PARK T G ET AL: "A new preparation method for protein loaded poly(D,L-lactic-co-glycolic acid) microspheres and protein release mechanism study" JOURNAL OF CONTROLLED RELEASE, NL, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 55, no. 2-3, 13 November 1998 (1998-11-13), pages 181-191, XP004143513 ISSN: 0168-3659 page 181, left-hand column, line 1 -page 182, left-hand column, line 3 page 182, right-hand column, paragraph 2 -page 183, left-hand column, paragraph 2</p>	1-3,7-10
X	<p>WO 98 46212 A (AMGEN INC) 22 October 1998 (1998-10-22) page 5, line 29 -page 6, line 30 page 9, line 3 - line 30 page 11, line 13 -page 12, line 30 page 13, line 3 - line 8; claims 1-4,11-17,20; examples 1,3-7</p>	1,2,6,7
A	<p>US 5 817 343 A (BURKE PAUL A) 6 October 1998 (1998-10-06) column 2, line 26 -column 3, line 26 column 4, line 57 -column 5, line 3 column 5, line 48 - line 67 column 7, line 16 - line 23; claims; example 1</p>	1-12
	-/-	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/29257

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>WO 00 38651 A (AMGEN INC) 6 July 2000 (2000-07-06) page 1, paragraph 2 page 2, paragraph 2 -page 3, paragraph 1 page 7, paragraph 2 page 14, last paragraph -page 15, paragraph 2; claims 1,10; example 9</p>	1-12

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 00/29257

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9701331 A	16-01-1997	AU 6242096 A CA 2224381 A EP 0835101 A JP 10007583 A	30-01-1997 16-01-1997 15-04-1998 13-01-1998
WO 9511009 A	27-04-1995	AT 175110 T AU 8017494 A CA 2172508 A DE 69415684 D DE 69415684 T DK 724433 T EP 0724433 A JP 9504026 T US 6080429 A	15-01-1999 08-05-1995 27-04-1995 11-02-1999 10-06-1999 30-08-1999 07-08-1996 22-04-1997 27-06-2000
WO 9846212 A	22-10-1998	US 6020004 A AU 7134898 A EP 0975334 A	01-02-2000 11-11-1998 02-02-2000
US 5817343 A	06-10-1998	AU 718866 B AU 2751797 A CA 2253667 A EP 0914095 A JP 2000513333 T WO 9742940 A	20-04-2000 05-12-1997 20-11-1997 12-05-1999 10-10-2000 20-11-1997
WO 0038651 A	06-07-2000	AU 2483800 A AU 2708100 A WO 0038652 A	31-07-2000 31-07-2000 06-07-2000

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.